Effect of Staining Procedures on the Results of Micronucleus Assays with Exfoliated Oral Mucosa Cells

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Abstract

Micronuclei in exfoliated epithelial cells are widely used as biomarkers of cancer risk in humans. To elucidate the effect of different staining procedures on the outcome of such investigation, we conducted a study in which the micronuclei frequencies in oral mucosa cells of heavy smokers (n = 20) and nonsmokers (n = 10) were evaluated with nonspecific (Giemsa, May-Grünwald-Giemsa) and DNA-specific (4,6-diamidino-2-phenylindole, Feulgen, acridine orange) stains, whereas with Giemsa-based stains, the frequencies of micronuclei in smokers were significantly (4- to 5-fold) higher in the smokers group, no significant increase was observed with any of the DNA-specific stains. Furthermore, the evaluation of cells of the two study groups with Feulgen stain showed that oral mucosa cells from smokers had significantly increased levels of nuclear anomalies other than micronuclei.

Introduction

About 25 years ago, Stich et al. (1) developed a protocol for micronucleus assays with exfoliated buccal mucosa cells, which was widely used in occupational and lifestyle studies (for reviews, see refs. 2 and 3). It was repeatedly emphasized that this noninvasive method might be a suitable biomonitoring approach for the detection of increased cancer risk in man because >90% of all human cancers are of epithelial origin (4). Approximately 200 studies with epithelial cells have been published in the last 25 years and apart from mouth cells, cells from other organs, such as nasal mucosa, cervix, bladder, esophagus, and bronchi, were also used (2, 3).

It was soon realized that strong interlaboratory and intralaboratory variations exist in micronucleus studies with exfoliated cells and efforts were made to standardize the assay. For example, Tolbert et al. (5) and Sarto et al. (6) defined morphologic scoring criteria for micronuclei and other confounding factors, such as age and gender of the study groups. However, only little attention has been, until now, given to the effect of different staining procedures on the results of micronuclei assays. An evaluation of the literature shows that a variety of different stains is used in micronucleus studies. Among the DNA-specific stains, the ones most widely used are Feulgen and acridine orange; in some experiments, 4,6-diamidino-2-phenylindole (DAPI) and propidium iodide were also used. About 30% of the studies in epithelial cells were conducted with nonspecific stains (Giemsa, May-Grünwald-Giemsa, and less frequently orcein). According to our knowledge, only one study (7) has been published in which the effect of different stains, namely Giemsa, Feulgen, Hoechst 33342, and propidium iodide, on the results of micronuclei experiments, was compared in cells from patients with different pathologies of the oral mucosa. Casartelli et al.’s findings indicate that the micronuclei frequencies are higher with Giemsa; however, no firm conclusions can be drawn from this study as no control group was included, the samples were collected from heterogeneous groups, and Hoechst 33342 and propidium iodide have rarely been used in micronuclei assays.

The aim of the present study was to further investigate if, and to what extent, different stains have an effect on the results of micronuclei studies in exfoliated cells. Therefore, buccal mucosa cells were collected from nonsmokers and heavy smokers, and the micronuclei frequencies were comparatively evaluated with two of the most commonly used DNA non-specific stains (May-Grünwald-Giemsa and Giemsa) and with three specific stains (Feulgen, acridin orange, and DAPI). Smokers were included as it is well established that they have increased risks for oral and other forms of epithelial cancer (8-10) and a number of studies on micronuclei induction in buccal cells of smokers has been published, which gave highly inconsistent results (refs. 11-31; for review, see ref. 28). Damage of epithelial cells leads to nuclear abnormalities other than micronuclei (metanucleated cells; ref. 32), such as karyorrhexis, karyolysis, pyknosis, condensed chromatin, broken eggs, binucleates, and it has been stressed that these abnormalities are difficult to distinguish from classic micronuclei and may lead to misinterpretations (5, 32). Therefore, we additionally evaluated these variables in both study groups and analyzed if the micronuclei frequencies scored with DNA-specific and nonspecific stains correlate with these anomalies.

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Materials and Methods

Participants. In total, 10 nonsmokers and 20 heavy smokers participated in the study. All subjects were healthy and none of them was exposed to DNA-damaging cytostatic drugs and ionizing radiation. All of them consumed a mixed diet and had moderate alcohol consumption (<1,000 mL beer or 400 mL wine/d).

Other characteristics of the two study groups are listed in Table 1. The number of cigarettes indicated is the average amount consumed per day during 3 months before the experiment was started. Informed consent was obtained from all participants concerning their participation.

Sample size was determined based on power considerations. Assuming a Poisson distribution of the number of micronucleated cells (33) and introducing a square-root transformation, a SD of ~0.55 resulted that was independent of the average number of micronuclei for the expected range of values. The power to detect a doubling of the average number of micronucleated cells at the 5% level of significance exceeds 90% for a 2:1 ratio of the number of subjects in the smokers and nonsmokers groups, respectively, if, overall, 30 subjects are included.

Sample Collection. Immediately before cell collection, the participants rinsed their mouths twice with tap water. Subsequently, the cells were scraped with wooden spatulas from each cheek, washed twice in plastic tubes (Sarstedt, Germany) containing 10 mL buffer solution [0.1 mol/L EDTA, 0.01 mol/L Tris-HCl, and 0.02 mol/L NaCl (pH 7.0)], and fixed in 80% cold methanol overnight (for details, see ref. 34).

Staining Procedures. Fifty microliters of the cell suspension were dropped onto wet cold glass slides and dried overnight in the dark at room temperature. From each sample, five to seven slides were prepared and stained. All stains were purchased from Sigma (Munich, Germany).

Giemsa stain (10%) was prepared in Sorensen's buffer (pH 6.8) as described by Vives Corrons et al. (35); the staining time was 20 minutes. For May-Grünwald, the cells were first stained in May-Grünwald solution for 3 to 5 minutes and subsequently with Giemsa (10%; see above) for 10 minutes (35). Acridine orange was dissolved in bidistilled water (0.01%); the staining time was 15 minutes (36). For DAPI, the stock solution was diluted (1 µg/mL) with bidistilled water; the staining time was 30 minutes (15). For Feulgen, the cells were placed in beakers with 5.0 mol/L HCl at room temperature for 15 minutes, rinsed with distilled water (15 min), and subsequently stained with Schiff's reagent for 90 minutes (37).

For direct comparisons of the results obtained with the different procedures, some of the Giemsa and May-Grünwald–Giemsa–stained slides were destained in methanol/glacial acetic acid (3:1) overnight (7) and subsequently stained with either acridine orange or Feulgen.

Evaluation of the Slides. From each participant, at least 1,500 cells were evaluated with each staining procedure. Micronuclei were scored in normal cells according to the criteria defined by Tolbert et al. (5). In addition, the frequencies of nuclear anomalies, namely karyolysis, pyknosis, karyorrhexis, broken eggs, and condensed chromatin, were recorded in Feulgen-stained slides as described by Tolbert et al. (ref. 5; for details, see also ref. 32). The analysis of the slides was carried out by two experienced scorers; micronuclei and metanucleated cells (i.e., cells with karyorrhexis, karyolysis, pyknosis, condensed chromatin, broken eggs, binucleates) were only registered after consensus.

Giemsa-, May-Grünwald-Giemsa–, and Feulgen-stained slides were evaluated under a light microscope (Nikon Eclipse E200) with 1,000-fold magnification using oil immersion. DAPI- and acridine orange–stained slides were evaluated under a fluorescence microscope (Nikon Microphot FXA, filters UV-2a, 330-380 nm and B-2A, 450-499 nm) with 1,000-fold magnification (oil immersion); according to the suggestion of M. Fenech (Commonwealth Scientific and Industrial Research Organization, Adelaide, Australia), Feulgen-stained slides were additionally scored under a fluorescence microscope (filter G-2A, 510-560 nm).

Statistics. A square-root transformation was chosen and homogeneity of variance of transformed values was tested by Bartlett's test. Differences between heavy smokers and nonsmokers as well as staining methods were tested by two-factor ANOVA with one between-subjects and one within-subjects factor. For each staining method, the difference between smokers and nonsmokers was tested by linear contrasts. The same methods were applied for comparison of Giemsa-stained and destained preparations. Age and alcohol consumption were included as covariables, and gender was added as a between-subject factor to analyze whether these variables contribute to differences between micronuclei frequencies. For comparison of cell anomalies between heavy smokers and nonsmokers, Wilcoxon's rank-sum test was applied. Results are expressed as prevalence ratios (ratio of the mean number of deviating cells per 1,000 cells) and their confidence intervals were computed based on Fieller's theorem.

Spearman rank correlation coefficients were computed to assess correlations between cell anomalies and average number of micronucleated cells from DNA specific and nonspecific staining.

Results

Figure 1 summarizes the micronuclei frequencies found in heavy smokers and nonsmokers with the different staining procedures. It can be seen that the micronuclei frequencies scored with Giemsa were significantly (~5-fold) higher in smokers than in nonsmokers; also, with May-Grünwald–Giemsa, the number of micronuclei was substantially (~4.5-fold) higher. On the contrary, no significant effects were observed with the DNA-specific stains. With acridine orange, the micronuclei frequencies were ~90% higher in smokers; with DAPI and Feulgen, the differences were 30% and 120%, respectively. When the Feulgen-stained slides were evaluated under fluorescence, an 89% increase was observed. It is also notable that the micronuclei frequencies recorded in the nonsmokers varied when different staining procedures were used; with nonspecific stains, the numbers were almost twice as high as with DNA-specific stains, but these differences failed to reach statistical significance. Age, gender, and alcohol consumption did not significantly affect the micronuclei frequencies with any of the staining methods and these variables had also no effect on the differences between smokers and nonsmokers.

Table 1. General characteristics of the participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Smokers</th>
<th>Nonsmokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. participants</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Age (mean ± SD, y)</td>
<td>36.1 ± 8.1</td>
<td>30.3 ± 8.7</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Males</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>No. consumed cigarettes/d (mean ± SD)</td>
<td>34 ± 11.9</td>
<td>12.2 ± 4.0</td>
</tr>
<tr>
<td>Duration of smoking (mean ± SD, y)</td>
<td>24.5 ± 2.0</td>
<td>26.0 ± 2.3</td>
</tr>
<tr>
<td>Body mass index (kg/m², mean ± SD)</td>
<td>22.5 ± 2.5</td>
<td>21.5 ± 3.0</td>
</tr>
</tbody>
</table>
correlation was seen with age (specific stains. whereas no such associations were found with the DNA-chromatin, when nonspecific staining procedures were used, such as karyorrhexis, karyolysis, binucleates, and condensed it can be seen that increased micronuclei frequencies were the latter effect failed to reach statistical significance.

In smokers than in nonsmokers. In addition, higher numbers of condensed chromatin, were 146%, 350%, 117%, and 54% higher in smokers than in nonsmokers. F-fluorescence microscopy, L-light microscopy.

To further substantiate the differences between results obtained with DNA-specific and nonspecific stains, we destained May-Grünwald slides from six smokers and an identical number of nonsmokers and evaluated the frequencies after DAPI staining. The results are depicted in Fig. 2, and it is evident that, again, no significant effect was detectable with the DNA-specific stain, whereas a pronounced difference between smokers and nonsmokers was observed with May-Grünwald.

The photographs depict cells with micronuclei after May-Grünwald-Giemsa (Fig. 3A and C), which were restained either with Feulgen (Fig. 3B) or with DAPI (Fig. 3D). It can be seen that intracellular structures that are classified as micronuclei with the non-DNA-specific stain are not detected with the DNA-specific stain.

To find out if smoking induces nuclear anomalies other than micronuclei, we evaluated comparatively the frequencies of different types of metanucleated cells in the two study groups. The results are summarized in Table 2. Four types of anomalies, namely karyorrhexis, karyolysis, binucleates, and condensed chromatin, were 146%, 350%, 117%, and 54% higher in smokers than in nonsmokers. In addition, higher numbers of cells with pyknosis were also found in smokers, although this latter effect failed to reach statistical significance.

Table 3 depicts the results of Spearman correlation analyses; it can be seen that increased micronuclei frequencies were significantly associated with certain other nuclear anomalies, such as karyorrhexis, karyolysis, binucleates, and condensed chromatin, when nonspecific staining procedures were used, whereas no such associations were found with the DNA-specific stains.

Gender and alcohol consumption did not correlate with the induction of metanucleated cells, but a borderline significant correlation was seen with age (P = 0.05); however, this did not affect the differences between smokers and nonsmokers.

Discussion

Our findings show that the results of micronuclei assays in exfoliated oral mucosa cells of smokers and nonsmokers depend strongly on the staining methods. Although much higher micronuclei frequencies were found in smokers with the non-DNA-specific stains Giemsa and May-Grünwald, no significant differences were seen with acridine orange, DAPI, and Feulgen. These comparisons indicate that micronucleation formation in epithelial cells may be overestimated when non-DNA-specific stains are used. This is an important observation as numerous studies have been conducted in the last two decades with exfoliated cells in which Giemsa stains were used.

Our observation that micronuclei formation in oral cells is overestimated with Giemsa-based stains is also supported by the results reported by Casartelli et al. (7), who compared micronuclei numbers after Giemsa and propidium iodide staining in cells from patients with different pathologies of the oral cavity (leucoplakias, squamous cell carcinomas). They found a 3-fold higher frequency of micronuclei with Giemsa than with Hoechst 33258. Comparisons between Feulgen, Hoechst 33259, and propidium iodide showed that 3- to 4-fold higher frequencies are obtained with the latter stain.

Also, the evaluation of earlier studies concerning the formation of micronucleated cells in mucosa of smokers confirms our findings (the evaluation is based on computer-aided searches in medical databases, studies in which the number of participants and/or the number of cigarettes were not specified were not included). Although consistently positive results were obtained with Giemsa (20, 21, 29, 31, 38), negative results were obtained in most studies with DNA-specific stains. We found in total 24 articles in which negative results were obtained in smokers with Feulgen, whereas an increase in micronuclei frequencies was reported in six articles only (6, 22, 23, 39-41). One group reported a positive result (6) but failed to confirm it in a subsequent study (42). Also, with other DNA-specific stains (DAPI, acridine orange), mainly negative results were obtained (15, 18, 24, 30, 37). The effect of alcohol consumption, age, and gender on micronuclei formation has been studied in several earlier investigations, and the results are strongly controversial. Although some investigators (6, 13, 26, 27, 30) found no effect of alcohol on micronuclei formation, Gabriel et al. (31) showed a significant effect. Also, Stich and Rosin (26) reported on a pronounced synergistic effect of alcohol in combination with smoking, whereas in another study (25) even an inverse relation was found. Also, the effect of age on micronuclei formation in buccal cells is unclear. In five investigations, no effect was detected (6, 13, 27, 29, 30); in two, an increase with age (39, 40) and in another a decrease with age (25) were detected. Piyathilake et al. (40) reported that the frequencies of micronucleated cells are 2.8-fold higher in females after adjusting for race, age, smoking habit, and levels of selected
vitamin, whereas in a Brazilian study (27) the number of micronuclei was significantly (i.e., >3-fold) higher in males. In the present investigation, we found neither in smokers nor in nonsmokers a statistically significant effect of all these variables on micronuclei formation, but it is notable that all participants consumed only small amounts of alcohol if any and that the age range was quite narrow; that is, 36.1 ± 8.1 years in the smokers group and 30.3 ± 8.7 years in the controls.

Table 2 shows that metanucleated cells with anomalies other than micronuclei, such as karyorrhexis, karyolysis, binucleates, and condensed chromatin, are significantly increased in smokers. These anomalies reflect the consequences of cell injury, cell death, and mitotic errors (5, 32). Some of them [pyknosis (PN), condensed chromatin (CC), and karyolysis (KL)] are accompanied by apoptosis (5, 32), which may result from DNA damage. However, the same phenomena are also seen in cells undergoing necrosis (5, 32) and cannot be regarded as reliable markers for increased DNA damage and cancer risk. Only few studies have been published in which such anomalies have been recorded. Significantly increased frequencies were seen, for example, in snuff users, petrol-exposed workers, and also in alcoholic patients with oral carcinomas (5, 22, 25, 32). In all studies including ours, karyolysis was strongly increased compared with the control groups, whereas the pattern of induction of the other anomalies differed strongly. For example, binucleates were increased in smokers but not in snuff users, whereas pyknosis was only elevated in the latter group (5, 32).

The effect of gender, age, and alcohol consumption on the formation of metanucleated cells has not been investigated in

Table 2. Frequencies of anomalies in buccal cells per 1,000 cells of smokers (n = 15) and nonsmokers (n = 10)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Mean ± SE</th>
<th>Prevalence ratio</th>
<th>Two-tailed P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condensed chromatin</td>
<td>Smokers</td>
<td>9.52 ± 0.89</td>
<td>1.54 (1.08-2.18)</td>
<td>0.019820</td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>6.19 ± 0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binucleation</td>
<td>Smokers</td>
<td>7.02 ± 0.59</td>
<td>2.18 (1.56-3.03)</td>
<td>0.000385</td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>3.23 ± 0.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Karyorrhexis</td>
<td>Smokers</td>
<td>7.59 ± 0.68</td>
<td>2.47 (1.67-3.64)</td>
<td>0.000428</td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>3.08 ± 0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Karyolysis</td>
<td>Smokers</td>
<td>9.90 ± 1.16</td>
<td>4.50 (2.64-7.67)</td>
<td>0.000040</td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>2.20 ± 0.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyknosis</td>
<td>Smokers</td>
<td>2.23 ± 0.41</td>
<td>1.45 (0.83-2.53)</td>
<td>0.244072</td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>1.54 ± 0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broken eggs</td>
<td>Smokers</td>
<td>0.15 ± 0.06</td>
<td>0.74 (0.25-2.23)</td>
<td>0.617618</td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>0.20 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degenerative cells</td>
<td>Smokers</td>
<td>36.42 ± 1.49</td>
<td>2.22 (1.81-2.73)</td>
<td>0.000032</td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>16.39 ± 1.59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Average number of inspected cells per participant: 2,127 (smokers) and 2,140 (nonsmokers).

Figure 3. Photographs of exfoliated oral mucosa cells with micronuclei (magnification, ×500). A and C, May-Grünewald-Giemsa–stained cells. B and D, cells stained with Feulgen and DAPI. Arrows, cellular structures that are classified as micronuclei according to established scoring criteria.
detail according to our knowledge. Sarto et al. (6) analyzed the effect of alcohol consumption in combination with smoking on the formation of binucleated cells and failed to detect an effect. In the present study, we observed a weak association between the age of the participants and the overall frequency of metanucleated cell, but as stressed above, the design of our study does not allow drawing of firm conclusions.

It has been stressed by Tolbert et al. (5, 32) that these anomalies are sometimes difficult to interpret and may be misclassified as micronuclei. This assumption is supported by the fact that, in the present study, significant correlations were observed between micronuclei frequencies and the formation of cells with karyorrhexis, karyolysis, binucleates, and condensed chromatin with nonspecific stains (Table 3). Another possible confounding factor in micronuclei studies is the formation of keratin granules that are found in degenerated cells with nuclear anomalies (43). These round cytoplasmic bodies, which are formed as a consequence of cell injury (43), do not contain DNA and may be classified as micronuclei with nonspecific stains.

Degenerated cells with abnormal nuclei (other than micronuclei) are not only found in individuals exposed to toxic substances, but to a lesser extent also in nonexposed subjects (Table 2), and it is possible that the positive results obtained in some studies with smokers and nonsmokers (23, 39) in which significant differences were observed between these groups with Feulgen stain are due to misinterpretation of these structures as the micronuclei frequencies in the controls were unusually high in these investigations. As in most (>99%) earlier studies, the exfoliated cells were collected from both cheeks of each participant in the present investigation. Because our findings indicate that micronuclei studies with nonspecific stains reflect the levels of keratinization of the cells, it can be expected that strong site-specific effects are found when cells are collected from different areas as it is known the levels of karyorrhexis and keratinization in the oral cavity differ strongly. They are highest in the lips, whereas only low levels are found in the bottom of the mouth (13). Currently, Giemsa stains are also widely used in micronuclei studies with nonepithelial cells originating from a number of organs, e.g., in experiments with polychromatic erythrocytes in bone marrow of rodents (44), lymphocytes (45), splenocytes (46), and liver cells (47), and in in vitro studies with stable cell lines (48). Because the formation of keratin bodies and of the nuclear anomalies described above are an adaptive response to cell damage, which is only seen in (normally nonkeratinizing) epithelial cells (49, 50), it is likely that the overestimation of micronuclei formation with nonspecific stains is only relevant for experiments with epithelial cells, e.g., from the oral cavity, cervix, bladder, and esophagus. This assumption is supported by the findings of Surrales et al. (51), who compared micronuclei frequencies in human lymphocytes stained with DAPI and Giemsa and found even higher frequencies with the former stain.

Our investigation indicates that the results of earlier micronuclei studies in cells of epithelial origin should be interpreted with caution when Giemsa-based stains were used. Because our study and the majority of investigations concerning induction of micronuclei in smokers with DNA-specific stains yielded negative results and positive results may be due to misinterpretation, it is very likely that exposure of oral mucosa cells to genotoxic carcinogens, such as nitrosamines, polycyclic aromatic hydrocarbons, and many other compounds that are contained in tobacco smoke, does not lead to formation of micronuclei in the oral mucosa cells (8, 9). The increased risks of oral cancer in smokers may be due to acute toxic effects and inflammation that are not associated with micronuclei formation. Therefore, the use of micronuclei in exfoliated epithelial cells as biomarkers of exposure to lifestyle- and occupation-related genotoxic carcinogens warrants further evaluation in general.

Acknowledgments

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References


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